Research Focus

Knockdown stands up

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In the past year, the genetic research of mammalian cells *in vitro* has gained the advantages of RNA interference (RNAi), a process found in worms and plants by which double stranded RNAs mediate selective gene inactivation through mRNA destruction. Recently, two papers have shown that genes could be suppressed *in vivo* in mammals by RNAi, which has potential implications for both therapeutics and research.

Through the years, a vast array of versatile tools has been developed to ectopically express genes in mammalian cells, with applications ranging from basic cell-biology research to gene therapy. The powerful genetics of gene inactivation, however, remained restricted to organisms such as yeast, *Caenorhabditis elegans* and Drosophila. The advent of mouse knockout technology by homologous recombination gave rise to a large body of knowledge but has several limiting factors, including the necessity to obtain a physical copy of the relevant genomic region of the gene of interest, the relatively long time it takes to generate a knockout mouse and the restrictions associated with embryonic lethality. On top of that, although most research is dedicated to the (dys)functioning of human cellular physiology, in general the homologous recombination technology is unsuitable for use in human cells.

RNAi in mammals

The discovery of RNA interference (RNAi) whereby double stranded (ds) RNA molecules suppress the expression of complementary genes has endowed research with a whole new set of tools that facilitate genetic studies of somatic cells and could possibly be applied in the clinic. RNAi has been known for some years now in organisms such as *C. elegans* and plants [1,2]. However, a much wider interest in this phenomenon was sparked by the discovery that dsRNA can selectively suppress gene expression in somatic mammalian cells through RNAi [3,4]. Using short (<30 nucleotides) synthetic interfering RNA duplexes (siRNAs) it was possible to induce sequence-specific gene silencing yet avoid the non-selective toxic effects of long dsRNAs.

A major drawback of this technique was its transient nature owing to the limited availability and stability of the synthetic oligonucleotides. Gene expression was suppressed for a maximum period of one week. In the beginning of 2002, another important advance was made when several groups reported the construction of expression vectors that mediate the production of siRNAs from transcripts containing a stem and loop structure [5–7]. These vectors allowed the continued expression of siRNAs in the cells resulting in a persistent and specific alteration of the proteome of the target cell. This opened the door for studies of long-term loss-of-function phenotypes.

In vivo

Introduction of long dsRNAs into worms and plants, either by transgenes, injections, or feeding results in RNAi that is observed throughout almost the whole organism. However, until recently there have been no reports of *in vivo* use of RNAi in mammals. This gap has now been filled by two recent reports that show proofs of principle for the use of RNAi *in vivo* in mammals [8,9]. In the paper by McCaffrey *et al.*, synthetic siRNAs or a vector coding for siRNA against luciferase were co-transfected with a luciferase reporter construct using a hydrodynamic transfection method in which naked DNA was injected under pressure into the tail vein of a mouse. After 72 hours, the luciferase activity in the liver (the major recipient of DNA in these types of delivery methods) dropped by 75% for the synthetic oligonucleotides to 90% for the vector. Similar results were described in the paper by Lewis *et al.* in which synthetic siRNAs against luciferase or a secreted alkaline phosphatase efficiently reduced the expression of their respective reporter gene in the liver 24 hours after injections. This effect diminished over several days, reflecting the transient availability of the synthetic RNAs. Importantly, this paper further extends the observations by inhibiting the expression of an endogenously expressed gene. In the liver of green fluorescent protein (GFP) transgenic mice they observed a sizeable decrease in GFP expression after injection of GFP-targeting synthetic siRNAs, demonstrating that mRNAs transcribed from the host genome can be inhibited *in vivo* using this method.

It has been described that, in *C. elegans*, RNAi can spread between cells depending on the trans-membrane SID-1 protein [10]. SID-1 has homologs in mice and men, which raises the interesting possibility of systemic spreading of RNAi in mammals. In their paper, Lewis *et al.* show pictures of the GFP-targeted liver in which numerous single cells keep expressing GFP although they are embedded in between cells that lack expression of GFP because they were targeted by the siRNAs against GFP. If hepatocytes spread these siRNAs, a much less drastic contrast would be expected between cells that are and cells that are not targeted. Therefore, we can conclude from their observations that at least between human hepatocytes, the RNAi effect does not spread *in vivo*.

The paper by McCaffrey *et al.* also includes data suggesting that siRNAs can be used against viral targets

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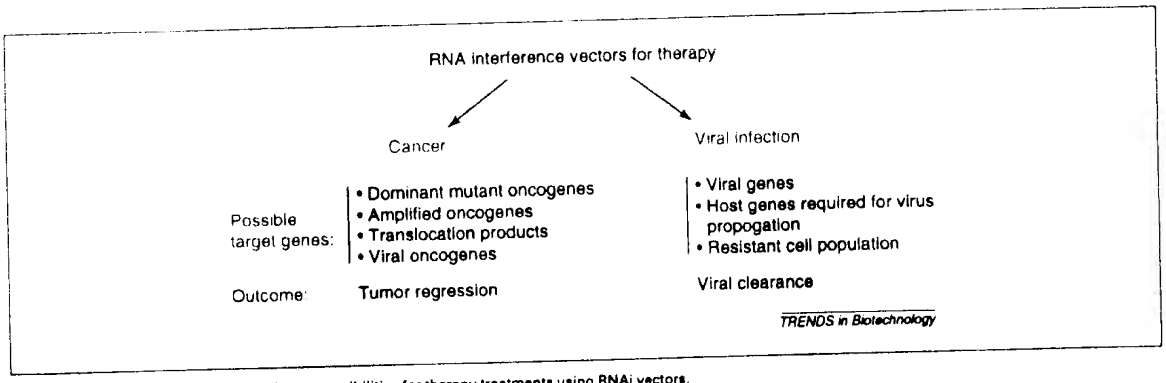

Fig. 1. A schematic model showing the future possibilities for therapy treatments using RNAi vectors.

[8]. Using a fusion of hepatitis C viral protein with luciferase, they show that they can repress its expression with specific siRNAs. This is still far from actually inhibiting the viral life cycle but several papers recently show *in vitro* inhibition of HIV and polio viral propagation using siRNA and make the treatment of viral infections by RNAi vectors possible [11–14].

New tools for therapy and research

A prerequisite for efficient and stable delivery of siRNA expression in cells would be the incorporation of its expression cassette in viral vectors. This is not a trivial condition because the major function of RNAi in plants and worms, and perhaps in mammals, is to suppress viral expression itself. However, using a self-inactivating siRNA-expressing retrovirus, we recently showed that such an approach is feasible [15]. We used the high sequence-selectivity of the siRNA system to target only the mutant oncogenic *RAS* allele in a human cancer cell line, leaving the wild-type *RAS* allele largely untouched. This resulted in a stable inhibition of *RAS* expression and loss of tumorigenicity. These data imply that RNAi can use retroviral vectors and possibly also other viral backbones *in vivo* to induce specific loss-of-function phenotypes. However, for cancer treatment even this delivery technique is most likely not efficient enough to target all cells of a tumor, even though the specificity of the RNAi allows for a rather unspecific delivery vehicle.

Perhaps a more promising therapeutic use for siRNA-expressing viruses is to create disease-resistant cells that will repopulate a diseased organ in a patient (Fig. 1). Protection can be achieved by suppressing the expression of viral genes or by inhibiting cellular proteins that allow viral entry or replication. The latter strategy is perhaps preferable because it avoids the notorious genetic instability of many viruses [12,13]. HIV-resistant bone marrow stem cells or hepatitis-C-resistant hepatocytes are two examples that are promising targets for such an approach in which the protected cells will gradually repopulate the organ and restore functionality. A recent paper showing that lentiviruses can transduce bone marrow cells *in vivo* [16] is particularly encouraging for this approach.

The observations that RNAi works *in vivo* in mammals not only allows the development of new therapeutical tools but also spawns the next generation of genetic research in mammals. For example, in mouse models it will be considerably easier to combine multiple knockdown constructs in one mouse to generate simultaneously multiple loss of function phenotypes in one generation. Moreover, because RNAi is highly selective, it should be possible to target specific splice variants, foreign or mutant genes by designing oligonucleotides derived from the unique sequences. With the complete sequence data from the human and mouse genome available it will be feasible to construct RNAi vectors against any gene in mind. Construction of an RNAi library targeting the whole genome will allow loss-of-function phenotypic screens in mammalian somatic cells or in whole animals. In contrast to the knockout technique, the vector-based RNAi systems achieve loss of function phenotypes without the loss of genomic information of the targeted gene. This leaves the possibility to restore the exact expression of the endogenous gene once the RNAi vector is silenced or removed. The vast majority of the mammalian RNAi systems are driven by the polymerase III promoter. This promoter can be manipulated such that it is susceptible to repression by the tetracycline-repressor system [17]. Therefore, the ability to turn the expression of a gene on-and-off at will, should allow experimental designs that thus far were unthought of. One such experiment could be to temporarily switch off a tumor-suppressor gene suspected to provide genome protection (e.g. a checkpoint gene) and then turn it on again. This will allow its requirement for either initiation or maintenance of tumorigenesis to be determined and whether or not it is a good target for late stage cancer treatments.

In conclusion, we have witnessed a fast and fruitful development of RNAi tools for *in vitro* and *in vivo* use in mammals. These novel approaches, together with future developments, will be crucial to put RNAi technology to use for effective disease therapy or to wield the awesome power of mammalian genetics.

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PNA and LNA throw light on DNA

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In some aspects, homogeneous (all-in-solution) nucleic acid hybridization assays are superior to the traditionally used heterogeneous (solution-to-surface) alternatives. Profluorescent probes, which reveal fluorescence enhancement or fluorescence polarization upon their binding to DNA and RNA targets, are a paradigm for the real-time sequence-specific homogeneous detection of nucleic acids. A variety of such DNA or RNA-derived probes of different constructs has already been developed with numerous applications. However, the recent additions to the field – locked nucleic acids (LNAs) and peptide nucleic acids (PNAs) – significantly increase the potential of profluorescent probes and provide a robust impulse for their new uses.

The DNA complementarity principle was discovered 50 years ago, along with the famous double-helical DNA structure [1]. This discovery soon led to several hybridization assays to detect and isolate specific nucleic acid sequences [2–4] – keystone procedures in molecular biology and biotechnology. Originally, the hybridization process was done as a homogeneous reaction in solution but the separation of hybrid molecules from unhybridized probes required either ultracentrifugation or chromatography and filtration: those who have worked with these early semi-homogeneous methods know how cumbersome and tedious those procedures are.

Consequently, a major advance was achieved when methods for immobilization and transfer of DNA to membrane or filter were developed [5–7]. One of the heterogeneous 'on surface' hybridization assays, Southern blotting [7], revolutionized the field and dominated for two decades. However, the need to immobilize hybrids on a solid surface inherent in the solid-phase hybridization formats limits their sensitivity (owing to non-specific adsorption of the probe) while the requirement to wash out unhybridized probes before detection of specific sequences prevents real-time monitoring.

These factors, and other drawbacks of non-homogeneous assays, drew attention to solution-based homogeneous hybridization formats. The study led to the first generation of profluorescent probes – DNA-derived molecular beacons (Fig. 1) [8,9], that turn brightly radiant under light after binding the target. These dual-labeled fluorogenic probes provide the means for truly homogeneous diagnostic assays and enable high-throughput multiplex analyses. But nothing is perfect: the novel hybridization tools are also not free from limitations such as insufficient stability, difficulties in the double labeling and lack of further signal amplification. The international search for better probes has recently led to new developments.

Singly labeled conformationally restricted fluorogenic probes

It is evident that singly labeled probes are more practical than dual-labeled probes because minimal DNA modification eliminates common problems caused by tagging two DNA sites, including low yield, high cost and singly labeled impurities. In the recent study from Caliper Technologies Corp. (Mt. View, CA, USA) the backbone-modified LNA (locked nucleic acid)-based probes were 5′ end-labeled with either rhodamine or hexachlorofluorescein and their hybridization to target DNAs was followed by measuring the fluorescence polarization (FP) of these dyes [10]. LNA, or locked nucleic acid, is a synthetic RNA derivative in which the ribose moiety in sugar-phosphate backbone is structurally constrained by a methylene bridge between

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